JOURNAL OF BACTERIOLOGY, Aug. 1972, p. 430-436

Vol. 111, No. 2
Printed in U.S.A.

Role of Iron and Sulfur in Pigment and Slime Formation by *Pseudomonas aeruginosa*[1]

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Received for publication 24 April 1972

Media and an analytical scheme have been developed which allow both a qualitative and quantitative estimation of the formation of pyocyanine, related phenazines, pyorubrin, and a blue and a yellow-green fluorescent pigment by *Pseudomonas aeruginosa*. Use of the defined pyocyanine medium of Frank and DeMoss with sulfate or various organic sulfur sources allowed formation of pyocyanine, related phenazines, and pyorubrin. When sulfite was the sulfur source with or without iron, *P. aeruginosa* formed either a yellow-green or a blue fluorescent pigment. Formation of fluorescent pigments of *P. aeruginosa* is related to the ability of sulfite to act as a specific sulfur source. In an investigation of the role of both added iron and sulfur sources, complex patterns of pigment formation were observed. In addition to the fluorescent pigments, sulfite also supported the formation of slime by *P. aeruginosa*.

As part of a recent study of tetrathionate enrichment broth (25), it was observed that certain sulfur salts altered pyocyanine production by *Pseudomonas aeruginosa*. A requirement for sulfur in the form of sulfate for pyocyanine formation by *P. aeruginosa* was first described by Thumm in 1895 (31), and this observation has been verified many times since. A requirement for sulfur generally in the form of sulfate has long been shown for fluorescent pigment formation. It became of interest to investigate the sulfur requirement for pigment formation by *P. aeruginosa*, especially in view of the fact that pyocyanine does not contain any sulfur and the fluorescent pigment is not known to contain any sulfur.

The requirement for minerals for pigment formation has long been known (31) and is well documented (3, 4, 11, 16, 17). The exact role of Fe, Mg, K, sulfate, and phosphate (3, 17) is not known, but their nature would suggest that they act as cofactors in the various enzymatic pathways of pigment formation. The data to be presented here suggest rather that added iron and sulfur sources control pigment formation in a complex fashion rather than acting as cofactors since various combinations of iron, sulfite, and sulfate allowed development of specific pigment patterns. Also, omission of both iron and the sulfur source allowed the development of another pigment pattern.

[1] A preliminary report of this study was presented at the 71st Annual Meeting, American Society for Microbiology, Minneapolis, Minn., 2-7 May 1971.

MATERIALS AND METHODS

Organism and medium. A laboratory strain of *P. aeruginosa*, NRRL B-4014, was grown in the defined pyocyanine medium of Frank and DeMoss (8). The medium contains, per liter: DL-alanine, 10 g; glycerol, 20 ml; K_2HPO_4, 0.139 g; $MgCl_2 \cdot 6H_2O$, 4.06 g; Na_2SO_4, 14.2 g (0.1 M); and ferric citrate, 0.1 g. The medium was used without *p*H adjustment, except where indicated; then the *p*H was adjusted with 1 N NaOH or 1 N HCl. The medium was used as described or modified either by changing the sulfur source added or by omitting various components. When substituting, compounds were added at the same concentration as the original. Incubation was stationary at 30 C.

Growth (turbidity) was recorded as either plus or minus. Because phenazines (including pyocyanine) and the fluorescent pigment are secondary metabolites (36), the kinetics of primary growth were not measured. In most instances, the cultures were inoculated at zero time.

Viscosity measurements. When sulfite was the added sulfur source, *P. aeruginosa* produced a viscous slime. The viscosity of the slime was expressed as the slime index and was determined before centrifugation of the culture (Fig. 1) by measuring the length of time the culture took to drain from a 10-ml volumetric pipette divided by the length of time distilled water took to drain from the same pipette. An attempt was made to measure the viscosity of the slime material in the culture broth with a Brookfield viscometer. However, because the slime material

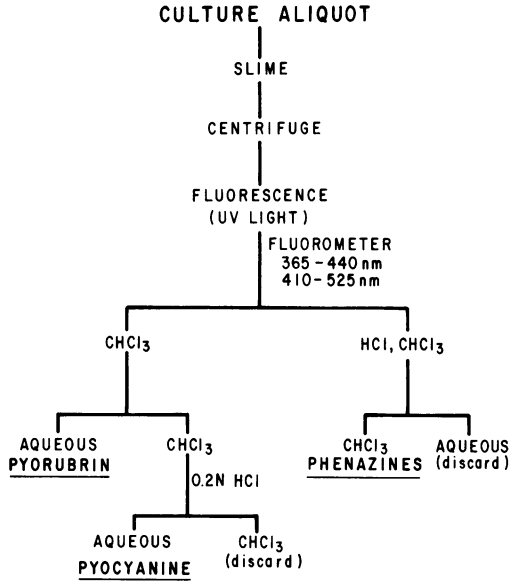

FIG. 1. *Scheme for analyses of pigments formed by Pseudomonas aeruginosa NRRL B-4014.*

wound around the spindle, accurate and reproducible viscosities could not be obtained.

Measurement of pigments. Pyocyanine, pyorubrin, and the yellow-green fluorescent pigment were differentiated by the characteristics of Meader, Leonard, and Robinson (24). Using their criteria, the analytical scheme given in Fig. 1 was developed. Analysis for the phenazine pigments (phenazine-1-carboxylic acid and oxychlororaphine) was developed with the criteria and characteristics of Chang and Blackwood (5), and these pigments were also included in the analytical scheme.

After centrifugation in the cold at 17,300 × *g* for 15 min the culture supernatant fluid was analyzed as indicated in Fig. 1.

Fluorescent measurements. At first fluorescence was determined by placing the culture under a Chromato-Vue (Ultra-Violet Products, Inc., San Gabriel, Calif.) and observing for fluorescence and its color.

Quantitative determination (relative intensity, RI) of fluorescence was made with an Aminco-Bowman spectrophotofluorometer at two settings: (i) an excitation wavelength of 365 nm and an emission wavelength of 440 nm, used to quantitate the blue fluorescent pigment, and (ii) an excitation wavelength of 410 nm and an emission wavelength of 525 nm, used to quantitate the yellow-green fluorescent pigment. Because RI of fluorescence is linear with concentration only in very dilute solutions (37), RI was read only in the range of 0 to 20. Solutions having an initial RI of 0 to 20 were also diluted to assure that no internal quenching of fluorescence occurred. If internal quenching was occurring, dilution of the sample would yield an increase in total RI (RI of sample times dilution factor); whereas, if there were no internal quenching, dilution of the sample would yield no change in total RI.

Pyocyanine. Two volumes of chloroform were added to one volume of culture supernatant fluid and shaken. The pyocyanine was then extracted from the chloroform into 0.2 N HCl and its absorbancy was measured at 520 nm (23) in a Beckman model B spectrophotometer against 0.2 N HCl.

Related phenazines. A 2-ml amount of concentrated HCl was added to 5 ml of culture supernatant fluid, and the solution was extracted with 10 ml of chloroform. The chloroform extract of the phenazines (phenazine-1-carboxylic acid and oxychlororaphine) was then clarified by filtration through filter paper, and the absorbancy of the filtrate was measured at 369 nm in a Cary model 14 recording spectrophotometer against chloroform. Since phenazine-1-carboxylic acid cannot be distinguished from oxychlororaphine at this wavelength, the results were expressed simply as phenazines absorbing at 369 nm.

Pyorubrin. Pyorubrin was determined on the aqueous fraction after extraction of the culture with chloroform. Its absorbancy was measured at 520 nm in a Beckman model B spectrophotometer against distilled water.

RESULTS

Influence of added sulfur source on pigments. The defined pyocyanine growth medium of Frank and DeMoss (8) contains Na_2SO_4 as the sulfur source. The results obtained when other sulfur sources are substituted for Na_2SO_4 are given in Table 1. The fluorescent pigment listed is the classical yellow-green one described by Meader, Robinson, and Leonard (24). However, negative cultures exhibited neither yellow-green nor any other visible fluorescence.

The data in Table 1 are only qualitative, but they do suggest that a major shift of pigments can be brought about by changing the sulfur source. One interesting aspect was the influ-

TABLE 1. *Influence of added sulfur source on pigment formation by Pseudomonas aeruginosa NRRL B-4014*

Sulfur source	pH	Pyocyanine and phenazines	Pyorubrin	Fluorescent pigment
Na_2SO_4	7	+	+	−
L-Methionine	7	+	+	−
L-Cysteine	7	+	+	−
L-Cystine	7	+	+	−
Na thioglycolate	7	+	+	−
Na_2SO_3	8.20	−	−	+
$Na_2S_2O_3 \cdot 5H_2O$	7	−	−	−
$Na_2S \cdot 9H_2O$[a]	7	−	−	−

[a] No growth. All other sulfur sources supported growth.

ence of sulfite. In another part of the study, it was observed that the addition of sulfite raised the *p*H of the medium to 8.20, and buffered the medium very strongly at this *p*H. When medium containing sulfite was adjusted to *p*H 7.0, neither fluorescent pigment nor growth was observed. The failure of the organism to grow is apparently due to the toxicity of HSO_3^- (27, 28). Also when the medium with sulfite omitted was adjusted to *p*H 8.20, no fluorescent pigment was produced although growth did occur.

The addition of sulfite to a culture after growth in sulfate does not cause the destruction of pyocyanine, and the addition of sulfate to a culture after growth in sulfite does not cause the loss of fluorescent pigments. If a sulfite-grown culture is adjusted to *p*H 7.0 with and without sulfite added after growth, fluorescence does not disappear. Thus, the pigments, once formed, are stable to the addition of a different sulfur source.

The *p*H of the normal medium with sulfate as the sulfur source is 6.9. When this medium was adjusted to *p*H 8.20, the organism produced the same pigments as at *p*H 6.9. During growth the organism will normally raise the *p*H of the medium. With sulfate present, the organism raised an initial *p*H of 6.9 to 8.7 in 13 days.

Figures 2 and 3 illustrate the kinetics of formation of pyocyanine and the yellow-green fluorescent pigment, respectively, when different sulfur sources are added to the medium. Pyocyanine is formed when Na_2SO_4 is present, but not with Na_2SO_3 or when the sulfur source is omitted although growth did occur (Fig. 2). Plots similar to Fig. 2 were obtained for the related phenazines and for pyorubrin.

Figure 3 illustrates the formation of the yellow-green fluorescent pigment with Na_2SO_3 as the sulfur source added but not with Na_2SO_4 or when the sulfur source was omitted although growth did occur.

Influence of added sulfur source on slime formation. One interesting observation of the influence of sulfur source on pigment formation was the production of a slime by *P. aeruginosa* when sulfite was the sulfur source (Fig. 4). The composition of this slime is not known, but it is thought to be a protein. It can be precipitated with ethanol or acetic acid and it gives a positive Lowry test for protein (22) and negative Molisch (6) and anthrone (34) tests for carbohydrate. The data in Fig. 4 indicate that *P. aeruginosa* has the ability to synthesize the slime material and then degrade it upon further incubation.

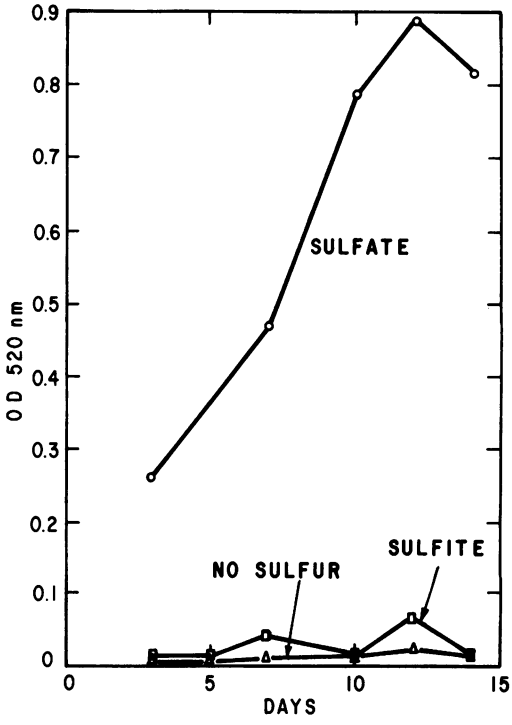

FIG. 2. *Influence of sulfur source added on pyocyanine formation by Pseudomonas aeruginosa NRRL B-4014.*

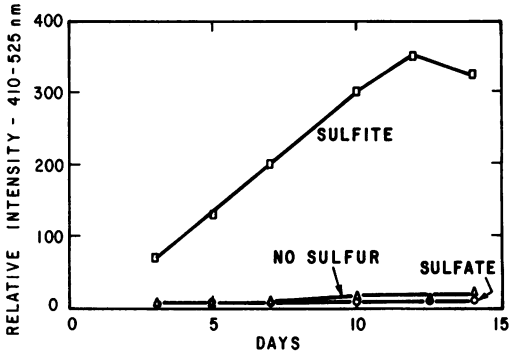

FIG. 3. *Influence of sulfur source added on the formation of the yellow-green fluorescent pigment by Pseudomonas aeruginosa NRRL B-4014.*

Influence of added sulfur and iron on pigment formation. Figure 3 shows that when sulfite was the sulfur source added to the complete medium including iron, fluorescent pigment was formed. This is an interesting observation since there are differences of opinion concerning the need for iron in fluorescent pigment formation. (i) Some investigators remove (26) or bind (10) the iron; (ii) others do

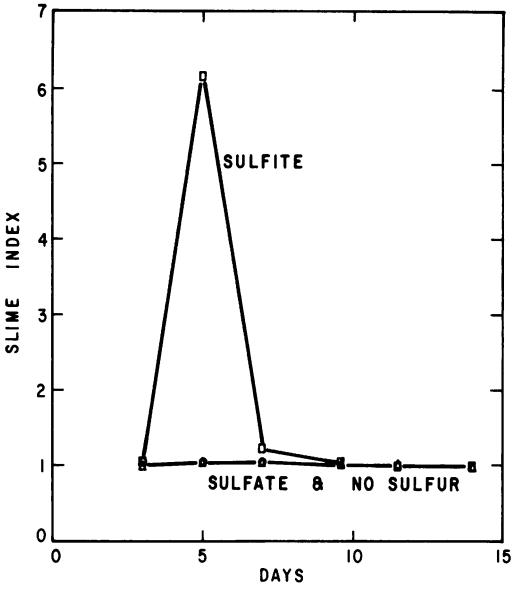

FIG. 4. *Influence of sulfur source added on slime formation by Pseudomonas aeruginosa NRRL B-4014.*

not add any iron (11, 15, 16, 18); and (iii) still others add small quantities of iron (4, 17, 19).

A culture containing sulfite but no iron gave a blue fluorescence instead of the typical yellow-green fluorescence observed when iron was present. Figure 5 shows the fluorescence of the two different pigments which are formed depending on whether or not iron is present. Growth occurred with all variables.

The properties, including the fluorescent characteristics, of the blue fluorescent pigment, suggest that it is a pteridine derivative (9, 12, 35, 37). In that pteridines are known to be metal chelators (1), it was thought that the yellow-green fluorescent pigment might represent an iron chelate of the blue fluorescent pigment. However, when iron was added to the blue fluorescent pigment, the spectral and fluorescent patterns did not change.

Because *p*H, sulfur source (sulfite and sulfate), and iron individually had various effects on the pigments formed by *P. aeruginosa*, it was of interest to combine all these variations in one study. These data are presented in Table 2. The readings are a combination of values obtained after 7 and 14 days of incubation. The different additions were considered negative for the specific pigment if the amount of pigment formed was at or below the base line amounts of the pigments (cf. Fig. 2, 3, and 5). Addition of iron alone to the basal medium allowed no pigments to be formed. Addition of sulfite allowed formation of the blue fluorescent pigment whereas sulfate allowed pyocyanine and phenazines. When iron was added to the media containing sulfite, the yellow-green fluorescent pigment was formed, whereas the addition of iron to the sulfate medium permitted *P. aeruginosa* to form pyorubrin in addition to pyocyanine and phenazines. However, with neither iron nor a sulfur source, all pigments except pyorubrin were formed. These last results suggest that pigment formation in *P. aeruginosa* is under complex control rather than the iron and sulfur source acting as cofactors.

The results in this table also illustrate the influence of sulfite and *p*H. Whenever sulfite is the sulfur source even in combination with sulfate and the *p*H is adjusted to 7.0, neither growth nor fluorescent pigment formation occurred. The production of both the blue and the yellow-green fluorescent pigments when both sulfite and sulfate but no iron are added to the basal medium may represent some form of control mechanism or possibly the presence of some intermediate level of iron due to contaminating components of the broth.

Replacement study. Cultures of *P. aeruginosa* were grown for 48 hr in broth with either sulfite or sulfate as the sulfur source. The cultures were then centrifuged at 17,300 × *g* for 15 min in the cold; the cells were washed once with sterile 0.1 M phosphate buffer (*p*H 7.5) and resuspended in media with either sulfate or sulfite as the sulfur source. Care was taken during the centrifugation and handling to minimize contamination of the cells. After 24 hr in the resuspension medium the cells had produced the pigment(s) of the resuspension media; cultures in sulfite formed fluorescent pigment, and cultures in sulfate formed pyocyanine, pyorubrin, and phenazines.

DISCUSSION

Considerable confusion exists in the literature concerning the naming of the fluorescent pigments produced by pseudomonads. Lehmann, Neumann, and Breed (20) proposed the term "bacterio-fluorescein" for these pigments. Later investigators (15–18, 32) have used the shortened term "fluorescein" or "fluorescin" to describe the fluorescent pigments produced by either *P. aeruginosa* or *P. fluorescens*. To avoid further confusion and to emphasize that these fluorescent pigments were different in spectral and fluorescent properties from fluorescein or fluorescin, or both, Elliott (7) proposed the term "pyoverdine." However,

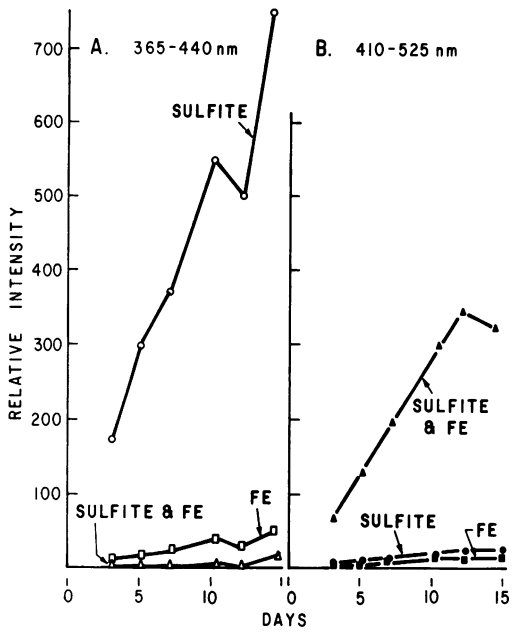

FIG. 5. *Influence of sulfite and iron addition on the fluorescence of the blue (A) and the yellow-green (B) fluorescent pigments by Pseudomonas aeruginosa NRRL B-4014. Basal broth contains glycerol, alanine, potassium phosphate, and magnesium chloride.*

in this paper, to avoid naming compounds whose structures are not known, these pigments have been referred to simply as the blue or the yellow-green fluorescent pigments.

The role of iron in fluorescent pigment formation is obscure. Weinberg (36) considers the fluorescent pigment as a secondary metabolite whose formation is under the control of iron. Other investigators (4, 21, 32) have observed an inverse relationship between fluorescent pigment and iron concentration. In this study iron controlled only the type of fluorescent pigment formed when sulfite was the sulfur source.

The role of added iron and sulfur sources is not known. The data in Table 2 suggest complex cellular control of pigment formation. Frank and DeMoss (8) proposed that the levels of sulfur compound seemed to represent a combination of sulfur requirement and ionic strength effects. The data in Table 2 suggest neither of these possibilities. This is especially evident when both iron and sulfur source are omitted; all pigments except pyorubrin are formed.

The *p*H influence on fluorescent pigment was observed by Sullivan (28), who found that an alkaline reaction favored its formation. This may explain why many of the media devised to detect fluorescent pseudomonads (11, 15, 19,

TABLE 2. *Influence of added iron and sulfur sources and pH on pigment formation by Pseudomonas aeruginosa NRRL B-4014*[a]

Additions and modifications				Pyocyanine	Pyorubrin	Phenazines	Fluorescent pigment		Growth
Iron	Sulfite	Sulfate	*p*H				Blue 365/440	Yellow-green 410/525	
−	−	−	6.9	+	−	+	+	+	+
−	−	−	8.2	+	−	+	+	+	+
+	−	−	6.9	−	−	−	−	−	+
+	−	−	8.2	−	−	−	−	−	+
−	+	−	7.0	−	−	−	−	−	−
−	+	−	8.2	−	−	−	+	−	+
−	−	+	7.0	+	−	+	−	−	+
−	−	+	8.2	+	−	+	−	−	+
+	+	−	7.0	−	−	−	−	−	−
+	+	−	8.2	−	−	−	−	+	+
+	−	+	7.0	+	+	+	−	−	+
+	−	+	8.2	+	+	+	−	−	+
−	+[b]	+[b]	7.0	−	−	−	−	−	−
−	+[b]	+[b]	8.2	−	−	−	+	+	+
+	+[b]	+[b]	7.0	−	−	−	−	−	−
+	+[b]	+[b]	8.2	−	−	+	−	+	+

[a] Basal broth contained glycerol, alanine, potassium phosphate, and magnesium chloride.
[b] 0.05 molar.

32, 33) contain no carbohydrate but rather an amino acid as both carbon and nitrogen source. Contaminating organisms would ferment any carbohydrate present to acid end products and hinder fluorescent pigment formation. Also the fluorescence of many compounds is decreased at acid *p*H values.

One early objective of this study was to determine what steps in pyocyanine formation sulfate affected. This pigment pathway was chosen because many of the steps in it are known (14). The replacement study in which the cells produced the pigments of the resuspension medium suggested several possibilities. (i) The intermediates for the pigments are already present in the cells before resuspension; (ii) the early intermediates of the different pigments are the same; and (iii) there are similar pathways leading to the different pigments or at least parts of the pathways are similar. However, because of the complex pigment patterns that develop with different additions, and especially when both iron and the sulfur source are omitted (Table 2), it may not be possible to determine the step(s) where these factors are involved or even to isolate or measure the intermediates that accumulate with the additions or omissions.

In two instances addition of iron resulted in a simple direct change in the pigment pattern. In the first, the blue fluorescent pigment is formed with sulfite alone whereas the yellow-green fluorescent pigment is formed with sulfite and iron. Although the chemical structure of the yellow-green fluorescent pigment is not known, it could easily represent the blue fluorescent pigment (which appears to be a pteridine derivative) with a different group at a side position. In the second instance, pyocyanine and related phenazines are formed with sulfate alone. With both iron and sulfate, pyorubrin is formed. The structure of pyorubrin is not known, but it could represent some modification of the phenazine nucleus. Both pyorubrin and acid pyocyanine have an absorption maximum at 520 nm, suggesting that they may have similar or at least related structures. However, pyorubrin is only soluble in water, whereas the phenazines are soluble in both water and chloroform.

The production of slime is considered to be a characteristic of *P. aeruginosa* (13). The exact composition of the slime is not known, but it apparently varies from strain to strain (2). In the present study (Fig. 4) slime was found to be formed under specific cultural conditions, i.e., when sulfite was the sulfur source added. Slime was formed and degraded in the same manner with or without iron. Further studies on the slime should include a subunit and an elemental analysis. Since sulfite is an absolute requirement for slime formation, it would be of interest to see if there is any sulfur covalently bound to the primary structure of the slime polymer.

Two possible applications can be suggested. First, there is a correlation between an increase in fluorescent pseudomonads and the spoilage of fresh meat (29). Based on this study, plating media with sulfite and iron could be developed which would permit better and faster detection of this important group of bacteria. Second is the use of *P. aeruginosa* with the described media to prepare large quantities of fluorescent pigments for other studies. In this system, the pteridines are present in the culture broth, and extraction of the cells and its accompanying problems are avoided. Specific pteridines have been implicated as cofactors in several enzymatic reactions (9, 12), and a ready source of these pteridines would allow in vitro study of the reactions.

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